COMPLEMENT FIXATION WITH ACID-FAST BACTERIA

III. IN TUBERCULOSIS

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Complement fixation with serum from tuberculosis and antigens of tubercle bacilli or their products, has been the subject of a number of investigations. No other bacterial antigen has been so widely used in testing human serum. The earlier work, after the first use of the test by Widal and Le Sourd[1] in typhoid fever and Bordet and Gengou[2] in tuberculosis, was somewhat contradictory and was received with much skepticism because of the lack of conformity between the clinical cases and the fixation tests. It is believed that these earlier discrepancies may have been due to the imperfect knowledge of some of the technical difficulties of the fixation reaction and also to the nature of the antigens used. Within the past few years, however, increasing numbers of reports have appeared in which the results of the tests have corresponded closely to the clinical analyses of the cases so that confidence in the diagnostic value of the test in tuberculosis is increasing. Many who have studied the reaction feel that its value when properly done is quite comparable to that of the Wassermann reaction in syphilis.

A review of the work already reported, which comprises more than one hundred articles, will not be given here since a summary of the more important papers may be found in recent publications of Craig,[3] Miller,[4] Moon,[5] Bronfenbrenner,[6] Stoll and Neumann,[7] and others. It may be stated that the antigens that have proved most satisfactory are culture filtrates and emulsions of the tubercle bacillus. A high percentage of positive reactions in known clinical tuberculosis has been the rule with the use of these antigens. One discouraging feature in the use of the test is a nonspecific fixation that has been noted in cases not clinically tuberculosis, particularly in syphilitics with strongly positive Wassermann reactions. The number of syphilitics giving such positive reactions has varied, but has frequently been from 20 to 40%. This has led to a number of modifications in the antigen in order to secure preparations that would not react with serums from syphilitics and yet give fixation with a large percentage of cases of tuberculosis. The more recent reports

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[1] Compt. rend. Soc. d. biol., 1901, 53, p. 841.
[2] Compt. rend. Acad. d. sc., 1903, 137, p. 351.
[3] Am. Jour. Med. Sc., 1915, 150, p. 781; Jour. Am. Med. Assn., 1917, 68, p. 773.
[4] Jour. Am. Med. Assn., 1916, 67, p. 519; Jour. Lab. and Clin. Med., 1916, 1, p. 816.
[5] Jour. Am. Med. Assn., 1918, 71, p. 1127.
[6] Arch. Int. Med., 1916, 17, p. 492.
[7] Jour. Am. Med. Assn., 1919, 72, p. 1043.

indicate that several of the antigens in use are capable of giving from 85 to 95% of positive reactions with cases of clinical tuberculosis, while less than 10% of syphilitics react positively, or no more than can be assumed to have a coincident tuberculosis. No antigen has yet been described, however, that can be considered an entirely satisfactory preparation and none gives uniform results in the hands of every investigator. Possibly this is due in part to a lack of standardization of the technical methods used in performing the test, and probably also to the lack of keeping qualities of some of the antigens used.

It is this inability on the part of several workers to verify the more favorable results with the test even when using the same or similar antigens that has prevented a more widespread confidence in its value. The explanation of the disparity in results in these cases is not clear. One point, however, may be mentioned regarding the instability of bacterial antigens. Only those who have had a considerable personal experience with antigens of this type realize the exacting care necessary to preserve their efficiency. Bacterial contamination, for example, not only leads to the appearance of anticomplementary substances, but such contaminated antigens may also give nonspecific fixation, or "false positives." Deterioration in fixing quality of an antigen frequently occurs after a time, and must be detected immediately. Constant and careful control of a bacterial antigen, therefore, is necessary to obtain comparable and reliable results.

Some investigators have attempted to determine the relation of the complement-fixation test to the clinical activity of the disease. The results show that while the clinically active cases give a very large proportion of positive reactions, the inactive or arrested cases vary from 9% (Miller[4]) to 61% (Brown and Petroff[8]). The value of such studies is considerably lessened by the difficulty in classification of lesions by clinical examination as active or inactive. Although it is possible to demonstrate definite clinical signs indicating an active pathologic process in one group, the other group of "inactive," "arrested" or "latent" tuberculosis may be expected to contain cases with active lesions not evidenced by clinical signs of activity. It is possible that a positive complement-fixation test may indicate an active or potentially active pathologic lesion but confirmation of such a belief must depend on other factors than clinical activity.

This paper deals with complement-fixation tests with serums from patients with tuberculosis using a number of acid-fast bacterial antigens, together with similar tests in syphilis and a variety of other diseases. As in the preceding experiments with immune rabbit serums and leper serums, the concentration of the complement-fixing antibodies has been studied by titration.

METHODS

The antigens used have been identical with those employed in the study of leper serums and the technic of performing the tests also has not varied from that described previously in connection with the same experiments. Since the titration of the serums was made by using decreasing amounts from 0.2 c c, an additional control or natural hemolysin was added for each serum. This consisted of 0.1 c c of serum with 2 units of complement, incubated with the other tests. At the end of 45 minutes, unsensitized sheep cells were added to this tube. Fifteen minutes later, when the tests were removed from the water

[8] Am. Rev. Tuberc., 1918, 2, p. 525.

bath, this control tube showed complete hemolysis in about one third of the serums tested. In such cases unsensitized cells only were added to those tubes containing 0.1 and 0.2 c c of that serum, since sufficient natural hemolysin was present for the hemolytic system. Cells sensitized with 2 units of hemolysin were added to all tubes containing less than 0.1 c c of serum and in all cases where 0.1 c c of serum did not contain enough natural sensitizer to lake the cells completely in 15 minutes. By this method the danger of any large excess of hemolysin was minimized. The absorption of natural hemolysin from all serums before testing them is somewhat tedious and does not seem necessary.

The serums tested were from several sources, most of them from patients in the University of California Hospital, and the San Francisco Hospital. The syphilitic serums, all of which gave strongly positive Wassermann reactions, were obtained through the kindness of Miss Miriam Olmstead of the Presbyterian Hospital and Dr. R. Ottenberg of the Department of Bacteriology, Columbia University, New York. Some of the serums from tuberculous cases were furnished by Dr. H. R. Miller of the latter laboratory. All serums were inactivated at 56 C. for 30 minutes and were tested within 1 week of collection from patients. The necessary controls were always included.

TABLE 1

COMPLEMENT FIXATION WITH VARIOUS ACID-FAST ANTIGENS AND SERUMS FROM TUBERCULOSIS

Antigens	Minimal Fixing Amount of Serum in c c						
	Generalized Tuberculosis in Monkey	Pulmonary Tuberculosis, Bacilli in Sputum	Tuberculous Glands of Neck	Tuberculous Epididymitis	Pulmonary Tuberculosis, Bacilli in Sputum	Tuberculous Spondylitis	Pulmonary Tuberculosis, Bacilli in Sputum
B. leprae—Duval (chrome)	0.006	0.006	0.008	.....	.....	.....	0
B. leprae—Levy	0.008	0.006	0.01				
B. leprae—B	0.008	0.004	0.006	0	0.004	.....	0
B. leprae—F	0.006	0.006	0.05				
B. leprae—G	0.006	0.008	0.03				
B. leprae—H	0.006	0.01	0.05				
B. leprae—Kedrowsky	0.006	0.004	0.01	0.05	0.004	0.004	0.2
B. leprae—Duval (nonchrome)	0.006	0.006	0.01	.....	.....	.....	0.2
B. leprae—Hansen (from skin)	0.008	0.004	0.008				
B. tuberculosis—human	0.004	0.004	0.008	0.03	0.004	0.004	0.2
B. tuberculosis—bovine	.....	0.004	0.006	0.05	.....	0.006	0
B. smegmatis	0.004	0.004	0.03	0.1	0.004	0.004	0.2
B. of butter	0.008	0.006	0.03	.....	0.006	.....	0
B. diphtheroid	0	0	0	0	0	0	0
Lipoid	0	0	0	0	0	0	0

0 means no fixation with 0.2 c c.

The results of the present series of tests have been arranged in 3 tables. Table 1 gives examples of the fixation test in tuberculous serums with a variety of acid-fast antigens; table 2 shows the reactions obtained with a number of cases of tuberculosis and a variety of other conditions, including syphilis; while table 3 illustrates the concentration of antibodies in syphilitic cases with a simple lipoidal antigen. In all instances the concentration of the fixing substances in the serums is given.

Although a considerable number of serums from cases of tuberculosis have been tested with several of the acid-fast antigens, the results have been so uniform that it seems unnecessary to show in detail more than a few typical examples. In table 1, tests of seven serums

with different antigens are given. All these showed active tuberculosis, 3 with pulmonary involvement, 3 in which bone, lymph node, and epididymis, respectively, were affected, and 1 of generalized tuberculosis in a monkey. The length of time required for titrating every serum with a number of antigens prevented the use of this procedure in a large series, but enough serums were tested to show that in tuberculosis, just as in leprosy and in rabbits immunized with acid-fast organisms, the serum contains substances that give fixation with members of the acid-fast group of bacteria. The reaction is not specific for the tubercle bacillus, but is an acid-fast fixation specific for the group of organisms. In a large percentage of the cases tested several antigens were tried, including a pigmented and a nonpigmented leprosy strain, a purely saprophytic organism like the smegma bacillus, and the tubercle bacillus. The variation of the different acid-fast antigens in their ability to give fixation with tuberculosis serums is slight, although, as with other serums, some strains are more constantly capable of fixing with smaller amounts of serum than others. Among the best of the antigens tested is B. tuberculosis (human). None of the serums studied showed a nonspecific fixation with an antigen prepared from a nonacid-fast diphtheroid bacillus. The difference in titer of the fixing bodies in different cases is striking and seems to bear no relation to the clinical type or severity of the disease.

Since the tubercle bacillus in all tests appeared to be one of the best of the acid-fast antigens, the results of its use in a number of cases are shown in table 2. In the 91 cases of tuberculosis, 90% gave positive fixation, 2 of 6 cases of suspected tuberculosis were positive, while 5 cases of healed pulmonary lesions were all negative. In a certain number of instances in this series, cases of "suspected tuberculosis," when found to have a positive acid-fast fixation, were subject to a more thorough clinical study which resulted in the demonstration of tubercle bacilli in the sputum. Fifty cases of acquired syphilis, all of which gave strongly positive Wassermann tests, showed 5 positive acid-fast fixations, a proportion slightly larger than the 5 positive cases of a group of 75 tests on persons with other diseases. This last group included cases of such acute infections as typhoid, scarlet and rheumatic fevers, pneumonia, diphtheria, endocarditis, pyogenic osteomyelitis, and malaria, as well as chronic processes like carcinoma, bronchial asthma, cardiorenal disease, various lesions of the central nervous system, chronic bronchitis and emphysema. The 5 positive reactions were given by patients with acute pulmonary infection

(influenza?) inoperable cancer of the stomach, chronic nephritis, polyserositis and by a healthy nurse on duty in a tuberculosis ward.

Among the tests is included a group of serums from various diseases received from a clinician who saw many cases of tuberculosis and was interested in the test, but somewhat skeptical about its value. These serums were marked by numbers only and the clinical diagnosis was not given until after the results of the tests had been reported. The high percentage of positive reactions given by cases of clinical tuberculosis convinced this physician that the test is of definite clinical value.

TABLE 2

COMPLEMENT FIXATION IN TUBERCULOSIS AND OTHER DISEASES WITH TUBERCLE BACILLI ANTIGEN, SHOWING CONCENTRATION OF FIXING BODIES IN THE SERUMS

Diagnosis	Total Number Tested	Number Negative	Minimal Fixing Dose of Serums in c c							
			0.2	0.1	0.05	0.03	0.01	0.008	0.004	0.002
Tuberculosis—pulmonary	78	8	4	17	18	14	6	5	5	1
Tuberculosis—lymph glands	6	1	2	1	..	..	1	1		
Tuberculosis—bones	6	0	..	3	2	..	..	..	1	
Tuberculosis—epididymis	1	0	..	..	..	1				
Tuberculosis—suspected	6	4	..	1	1					
Tuberculosis—healed	5	5								
Syphilis—Wassermann positive	50	45	2	2	1					
Various other diseases	75	70	1	3	1					

In all serums the smallest amount giving fixation with the antigen has been determined by titration, and the results (table 2) illustrate the relatively wide variation that may occur in the concentration of fixing antibodies in different serums. No relation could be shown between the titer of a serum and the clinical severity of the infection. It is very significant that such variation in titer does occur and in this may lie the explanation of the fact that certain cases of undoubted tuberculosis give negative reactions. In such instances, the concentration of antibodies is so slight that their recognition is not possible by the complement-fixation method, or the fixing substances may be entirely absent. When, for example, it is possible to obtain fixation with as little as 0.002 c c of serum in some cases with an acid-fast antigen, the reaction appears delicate and the antigen reliable. When other cases show fixation with only 0.02 c c or 0.2 c c, one is justified in saying that the reaction is as delicate and the antigen as reliable, but that less fixing bodies are present in such serums. A small group of cases in which too small an amount of fixing antibody is present for fixation in 0.2 c c must be considered an example of the limitation of the method. The discovery of an antigen preparation that will give positive reactions with every case is improbable.

A similar titration of the serums from 50 cases of syphilis with a simple lipoid antigen is given in table 3. One object of such a study was to determine whether any relation existed between the presence of a high concentration of syphilitic antibody in certain serums and a positive acid-fast fixation No definite relationship was found. Of the 5 serums that gave positive acid-fast fixations, the titer with lipoidal antigen was 0.004 c c in 3, while in the others it was 0.006 c c and 0.008 c c. Only strongly positive syphilitic serums were tested. An analogy between the fixation reaction of syphilitic serums with lipoidal antigen and acid-fast fixation in serums from tuberculosis may be pointed out by comparison of the titration data given. In both instances, the concentration of complement-fixing antibodies varies

TABLE 3

COMPLEMENT FIXATION IN SYPHILIS WITH SIMPLE LIPOIDAL ANTIGEN, SHOWING CONCENTRATION OF SYPHILITIC ANTIBODY IN SERUMS

Diagnosis	Number Tested	Minimal Fixing Dose of Serums in c c								
		0.05	0.03	0.01	0.008	0.006	0.004	0.002	0.001	0.0003
Syphilis—acquired.....	50	6	15	4	5	7	8	2	2	1

considerably in different cases. It has been suggested that certain cases of tuberculosis do not give a positive acid-fast fixation because of an unrecognizably small amount of antibody in the serum, and a similar lack of syphilitic antibody probably explains the negative Wassermann reactions in certain cases of syphilis. Although no such instances were recognized in this series, there seems to be no doubt that these cases are not infrequent. Warthin[9] has found at necropsy active syphilis with spirochetes in many cases giving negative Wassermann tests during life, and the frequency of negative blood Wassermann reactions in syphilis of the central nervous system is well known. Since complement-fixing bodies are apparently subject to considerable variation in concentration in the blood serum in both syphilis and tuberculosis, it is not surprising that certain cases contain insufficient amounts to be recognized.

Although most workers with the test in tuberculosis agree that the antigen should contain a number of strains of tubercle bacilli to obtain the best results, there is some evidence from these experiments that at least one other factor is important in the preparation of a good antigen. It has been mentioned previously that certain acid-fast

[9] Am. Jour. Med. Sc., 1916, 152, p. 508.

antigens are superior to others in fixing with smaller amounts of antibody in immune serums, and that these antigens give the most uniform suspensions in salt solution. In two instances only (B. smegmatis and B. tuberculosis) have different strains of the same organism been studied and in both cases the strain that gave the most even suspension made the better antigen. So consistently has this physical characteristic of the bacillary suspension been associated with the ability of an antigen to fix with small amounts of antibody in serum, that the property of forming a uniform, fine suspension in salt solution seems essential in the best antigens. Apparently, strains which contain a relatively large amount of waxy material do not lend themselves so well to saline suspension and form less delicate antigens. It seems quite possible that similar variations in different cultures of tubercle bacilli may be encountered.

The amount of antigen used in the test is of much importance, since large amounts of antigen tend to give nonspecific fixation irrespective of any anticomplementary action of the antigen itself. This is especially true with regard to syphilitic serums. Various bacterial cultures act as antigen in the Wassermann reaction if used in sufficient amounts and this is true also of tubercle and other acid-fast antigens. It is quite possible, however, to employ amounts of antigens which will give fixation with tuberculous serums, but which are insufficient to react with syphilitic serums. Such an adjustment of the proper antigen dose may be ascertained by preliminary titrations with several serums. As an additional safeguard, it seems advisable to include a control in each test with a simple lipoid antigen.

A method by which a comparison of antigens may be made is by titration of the minimal fixing dose of one or more serums. This shows which antigen is capable of giving fixation with the least amount of antibody, and seems preferable to the testing of fixed amounts of serums from a number of cases to determine which antigen reacts with the highest percentage.

The strength or intensity of the reaction with any serum is estimated more accurately by a titration of the serum than by an attempt to estimate the amount of hemolysis in a single tube, although such a titration involves additional time. The severity of the tuberculosis is not always indicated by the strength of the fixation, but those serums with a titer of less than 0.1 c c are practically always from cases that are clinically active. The large amount of blood necessary and the increased danger of anticomplementary action of the serum are among

the objections to using more than 0.2 c c as a maximum amount in the test. It is probable that some additional cases might give positive reactions with larger amounts of serum. Only relatively fresh serums or those that have been kept sterile and sealed should be used for the test. Serums that give negative reactions may become positive after bacterial contamination, and this change is associated with the development of anticomplementary action, since such serums if tested later are anticomplementary. Positive reactions obtained on old serums or those that have developed even slight anticomplementary properties are therefore unreliable. Complement-fixing substances do not disappear for many months, however, if serums are kept sterile.

The type of antigen most suitable for complement-fixation tests in tuberculosis is still somewhat in dispute. That used in these experiments has proved satisfactory, but no comparative study has been made with other antigens made from whole bacillary emulsions. Partial antigens and filtrates from cultures are open to certain theoretical objections since they do not contain all the antigenic substance. After extraction of bacilli with fat solvent, both the residue and the extract will act as antigen, and similarly both the filtrate (tuberculin) and the bacillary bodies from a broth culture have been used as antigens. In order to have all the antigenic substance possible it would seem of advantage to use whole cultures. The keeping qualities of dried antigens and the ease with which a standard suspension can be made, are distinct advantages.

SUMMARY

In tuberculosis, the serum contains complement-binding substances that give fixation when members of the acid-fast group of bacteria are used as antigen. These antibodies show a considerable difference in concentration in different serums, but this difference in titer does not correspond with the severity of the infection in the different cases. A relatively small percentage of patients with active tuberculosis have too small an amount of complement-fixing antibody to be recognized in the test. These cases are analagous to those of syphilis that give a negative Wassermann reaction. The test is of some value in calling attention to unrecognized tuberculosis, but does not always indicate a clinically active process.

Leprosy and tuberculosis are the only human infections that give the reaction, and since it is specific for the acid-fast group of bacteria, the term acid-fast fixation seems appropriate.